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Morphological and Molecular Revision of *Zoanthus* (Anthozoa: Hexacorallia) from Southwestern Japan, with Descriptions of Two New Species

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No clear method of identifying species in the zoanthid genus *Zoanthus* has been established, due in part to the morphological plasticity of this genus (*e.g.*, in polyp and colony form, oral disk color, tentacle number). Previous research utilizing the mitochondrial cytochrome oxidase I gene (COI) as a phylogenetic marker indicated that *Zoanthus* spp. in Japan may consist of only one or two species, despite a bewildering variety of observed morphotypes. Here we have utilized not only COI but also mitochondrial 16S ribosomal DNA (mt 16S rDNA) in order to clarify the extent of *Zoanthus* species diversity in southern Japan. Our molecular genetic results clearly show the presence of three monophyletic *Zoanthus* species groups with varying levels of morphological plasticity, including the new species *Z. gigantus* n. sp. and *Z. kuroshio* n. sp. We describe all three species found in this study, and identify potential morphological characters (coenenchyme and polyp structure as well as polyp external surface pigmentation patterns) useful in *Zoanthus* species identification. A morphological dichotomous key is provided to assist in field species identification.

Key words: mt 16S rDNA, COI, ITS rDNA, zoanthid, *Zoanthus*

INTRODUCTION

The encrusting anemone zoanthid genus *Zoanthus* (Lamarck, 1801) is distributed worldwide in shallow subtropical and tropical waters, and is commonly found on rocks and coral reef edges exposed to waves and/or currents. Although generally not dominant organisms in any given location, *Zoanthus* spp. are common in reef environments. Currently, 154 nominal species are reported according to the Hexacorallia database, but this number includes only four properly described species (Fautin, 2004). Recently, studies on *Zoanthus* spp. have been increasing in number. Examples include research on the evolution and characteristics of green fluorescent protein (GFP) (*i.e.* Gurskaya *et al.*, 2001; Labas *et al.*, 2002; Kelmanson and Matz, 2003) and the production of potentially antiosteoporotic alkaloids such as norzoanthamine (*i.e.*, Miyashita *et al.*, 2004), all of which utilized *Zoanthus* specimens from unknown or undescribed species. Thus, construction, validation, and utilization of a legitimate classification system for *Zoanthus* have gained new importance.

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Traditionally, *Zoanthus* and other zoanthids have been classified by their oral disk size and color (Uchida, 2001), polyp and coenenchyme characteristics (Fossa and Nilsen, 1998), and differences in their nematocyst structure (Ryland and Lancaster, 2003, 2004), as well as internal anatomy based largely on mesentery and septa (Herberts, 1987). However, no clear taxonomic system is in place, for several reasons, including polyp and colony morphological variation, the lack of a skeleton, a reliance on preserved samples, and a scarcity of thorough investigation (Fossa and Nilsen, 1998). As a result, while the genus *Zoanthus* is well established, classification and identification of species remains problematic and underreported.

Genetic methods are a potentially useful tool in helping discern phylogenetic and evolutionary relationships in cnidarians in general and zoanthids in particular. If recent genetic studies are any indication (Burnett *et al.*, 1995, 1997; Reimer *et al.*, 2004), single species of *Zoanthus* may encompass a wide variety of morphological types, and the true number of species may be grossly overestimated. For example, based on mitochondrial cytochrome oxidase I gene (COI) sequence data, it appears that four presumed species (*Z. sansibaricus, Z. gnophodes, Z. erythrochloros, Z. pacificus*) may actually be various morphotypes within the single species *Z. sansibaricus* (Reimer *et al.*, 2004).

To investigate *Zoanthus* species diversity as well as

answer lingering questions on the accuracy of COI as a species-level marker for zoanthids and *Zoanthus*, we collected a wide morphological variety of *Zoanthus* from several different sites in Japan and examined them both morphologically (using *in situ* images and microscopic analyses with cross-sections) and genetically (using COI and mitochondrial 16S ribosomal DNA (mt 16S rDNA)). Based on our sequence data and morphological results, we here treat three species in detail, revising *Z. sansibaricus* and describing two new species, and provide a morphological key for the identification of *Zoanthus* species found in southwestern Japan. We also propose a taxonomic system for future *Zoanthus* species identification based on genetic sequencing and polyp and colony morphological characters.

MATERIALS AND METHODS

Sampling

Samples of *Zoanthus* spp. were collected from several field sites in Japan (Kushimoto, Shirahama, Kokubu, Sakurajima, Bonotsu, Yakushima, Amami, Okinoerabu, Yoron, Kerama) between June 2003 and November 2005 and stored in 80–100% ethanol at –20°C. Samples were collected of a variety of observed *Zoanthus* morphotypes at each site across the full range of depth and micro-environmental distribution. Additionally, a sample of *Parazoanthus gracilis* was collected from Jogasaki, Izu, Shizuoka, Japan for use as an outgroup. As samples were collected *in situ*, photographs were taken to assist in identification and for collection of morphological data. Additionally, sampling data (depth, environment, date) were recorded.

Morphological analyses

Digital photographs of all *Zoanthus* specimens were examined, and the following morphological data collected: oral disk/polyp diameter, oral disk color(s), tentacle count, polyp and coenenchyme form, polyp color. Morphological examination of samples followed the procedure described in Ono *et al.* (2005), with samples fixed in Bouin's fluid and embedded in paraffin. Samples were cross sectioned into 8-μm thick sections, stained with Azan, and observed under the microscope. Data were collected on polyp structure, diameter, and height; mesogleal thickness; and number of mesenteries.

DNA extraction, PCR amplification, and sequencing

DNA was extracted from samples (5-20 mg) and the COI gene was amplified following procedures outlined in Reimer *et al.* (2004).

Mitochondrial 16S rDNA was amplified using a set of primers provided by Sinniger *et al.* (2005), with the following thermal cycle conditions: 40 cycles of 30 seconds at 94.0°C, 1 minute at 52.0°C, 2 minutes at 72.0°C followed by a 5-minute extension at 72.0°C.

COI and mt 16S rDNA amplification products were checked by 1.5% agarose gel electrophoresis. The PCR-amplified DNA fragments were sequenced with an ABI PRISMTM 3700 DNA Analyzer (PE Biosystems, Foster City, CA, USA) using a BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). The sequences were analyzed using GENETYX-MAC version 8.0 (Software Development, Tokyo, Japan) and DNASIS Mac v3.6 (Hitachi Software Engineering Company, Ltd., Tokyo, Japan).

Phylogenetic analyses

New sequences obtained in the present study were deposited in GenBank (accession numbers AB219182~AB219194). By using CLUSTAL X version 1.8 (Thompson *et al.*, 1997), the nucleotide sequences of the COI gene and mt 16S rDNA from *Zoanthus* species were separately aligned with COI sequences from *Palythoa* spp. from Amami (AB128895; Reimer *et al.*, 2004) and Yakushima (AB128896; Reimer *et al.*, 2004), and *Parazoanthus gracilis* (AB214178; Reimer *et al.*, unpublished data), and mt 16S rDNA sequences from *Palythoa caesia* (AF282931; Burnett, unpublished data) and *Palythoa caribaeorum* (AF282932; Burnett, unpublished data), respectively. *Zoanthus* spp. COI sequences obtained from previous studies (AB128893, AB128894, AB128897, AB128898, AB194014~AB194026, AB194028~AB194036 (Reimer *et al.*, 2004); AB214162~AB214177 (Reimer *et al.*, unpublished data); AF282933~AF282936 (Burnett, unpublished data); and AY049060 (Longo *et al.*, 2002)) were also included in the alignments. The alignments were inspected by eye and manually edited. All ambiguous sites in the alignments were removed from the data set for phylogenetic analyses. We generated two alignment data sets: 1) 596 sites for 47 taxa (the COI gene); and 2) 532 sites for 15 taxa (mt 16S rDNA). The alignment data are available on request from the corresponding author.

For the phylogenetic analyses of the COI gene and the mt 16S rDNA sequences, the same methods were independently applied. Maximum-likelihood (ML) analyses were performed using PhyML (Guindon and Gascuel, 2003). PhyML was performed using an input tree generated by BIONJ with the general time-reversible model (Rodriguez *et al.*, 1990) of nucleotide substitution incorporating invariable sites and a discrete gamma distribution (eight categories) (GTR + I + Γ). The proportion of invariable sites, a discrete gamma distribution, and base frequencies of the model were estimated from the dataset. PhyML bootstrap trees (500 replicates) were constructed using the same parameters as the individual ML trees.

The neighbour-joining (NJ) method was performed using PAUP* Version 4.0 (Swofford, 1998), with the Kimura-2 parameter model (Saitou and Nei, 1987). NJ bootstrap trees (1000 replicates) were constructed using the same model.

SYSTEMATICS

Family Zoanthidae Gray, 1840
Genus *Zoanthus* Lamarck, 1801

Type species. *Zoanthus sociatus* (Lamarck 1801).

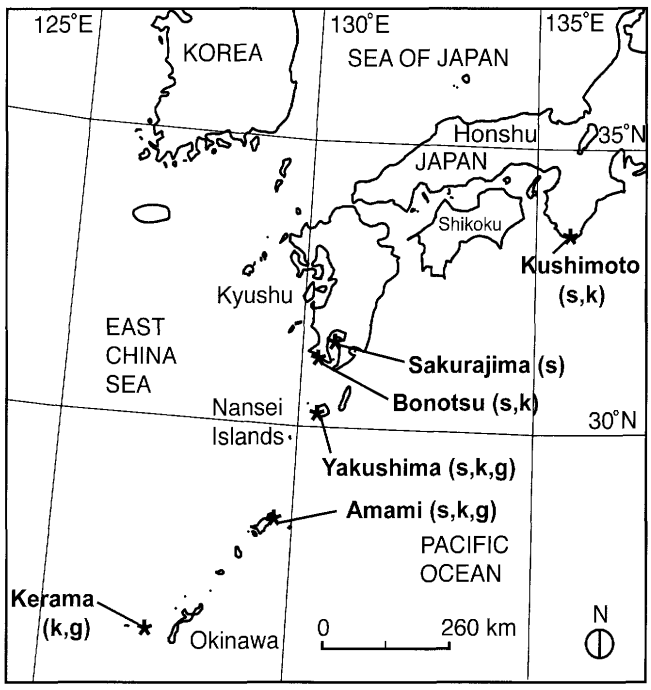


Fig. 1. Location of sampling sites in southern Japan, with indication of *Zoanthus* species found at each site. Abbreviations: s, *Z. sansibaricus*; k, *Z.kuroshio* n.sp.; g, *Z.gigantus* n.sp.

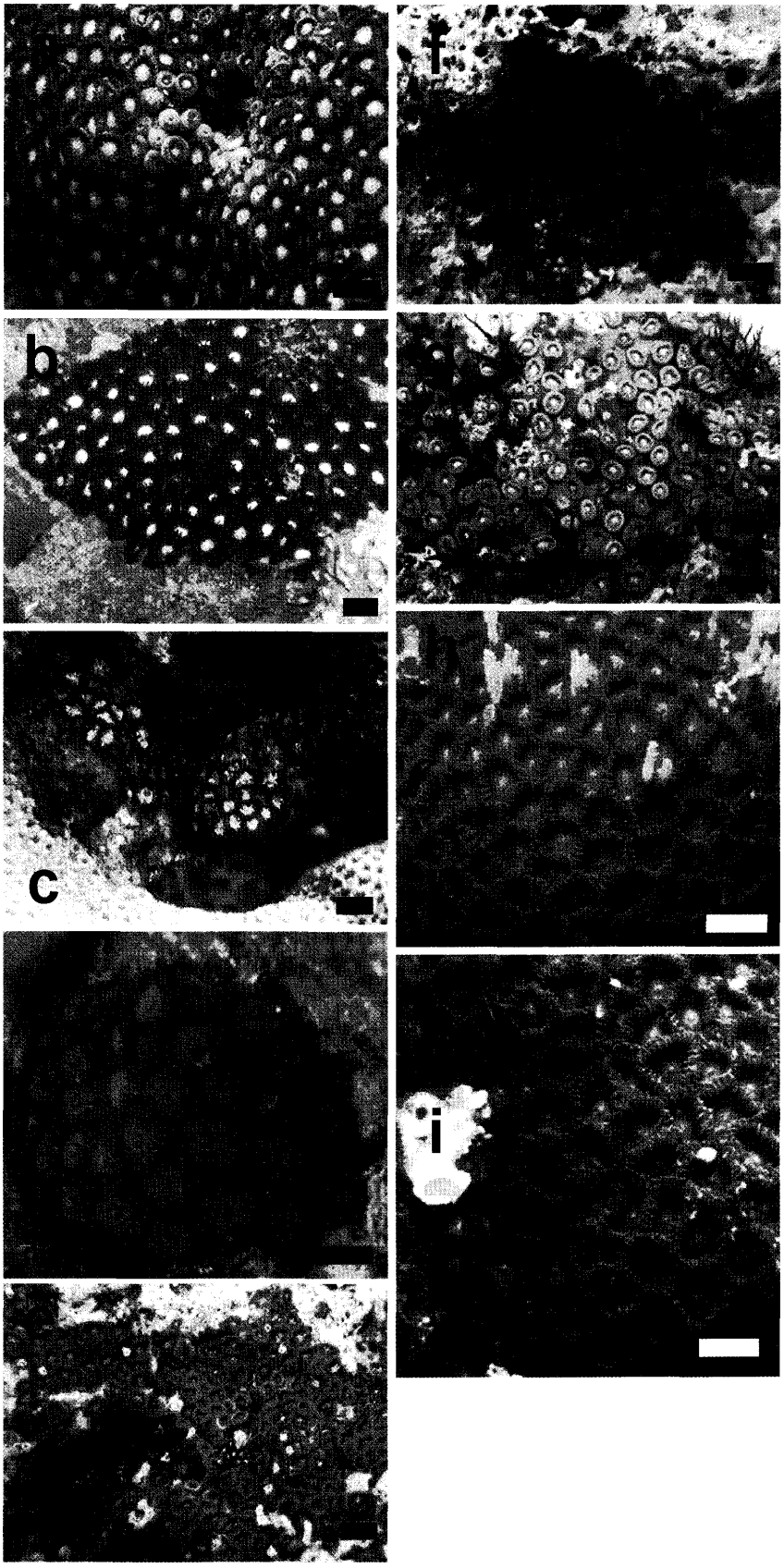


Fig. 2. *Zoanthus sansibaricus* samples of different morphotypes used in this study. **a)** sample ZSH2, collected from Sakurajima site, depth=–5.0 m. **b)** ZSH23, Sakurajima, –9.0 m. **c)** ZYS2, Yakushima, 0.0 m. **d)** SakZery1[1], Sakurajima, –3.0 m. **e)** ZAT1, Amami, 0.0 m. **f)** ZAT2, Amami, 0.0 m. **g)** ZAT3 (purple oral disk) and ZAT4 (yellow oral disk), Amami, 0.0 m. **h)** SakZgno[1], Sakurajima, –3.0 m. **i)** SakZsansi[1], Sakurajima, –3.0 m. White/black bars=1 cm. [1]Samples from Reimer *et al.* (2004). For sample abbreviations, refer to Table 1.

Diagnosis. Exist as clonal colonies with crowded polyps, can reach large (1.5 m^2) sizes, with no sand grains in the coenenchyme. Individual polyps erect and open in the daytime. Polyps usually 3–30 mm high, diameter 3–12 mm, expanded polyps up to 25 mm in diameter. Mesogleal sphincter muscle in two parts. Zooxanthellate. Generally believed to be hermaphroditic spawner, similar to most corals (modified from Fossa and Nilsen 1998).

Distribution and habitat. Worldwide in sub-tropical and tropical seas, in shallow waters (<30 m). Often found in the intertidal zone and tide pools, usually on rocks or coral-reef substrates

Remarks. General lack of detailed species descriptions and systematics. Many color morphotypes may exist in the same locale or even in intermingled colonies. Polyp size and colony shape can vary greatly with various microenvironmental conditions, such as water flow, *etc.* Colonies and polyps are hermaphroditic or gonochoric (Ryland, 1997).

Zoanthus sansibaricus **Carlgren, 1900**

(Figs. 1, 2, 5b, 6, 9; Tables 1, 2)

Voucher specimens. Voucher specimen 1, NSMT-CO 1441, collected from Hakamagoshi, Sakurajima, Kagoshima, Japan (30°35'N, 130°35'E), depth=3 m, on June 27, 2005 by SO; deposited in the National Science Museum of Tokyo (NSMT).

Voucher specimen 2, NSMT-CO 1442 (NSMT) Fort., collected from Hakamagoshi, Sakurajima, Kagoshima, Japan (30°35'N, 130°35'E), depth=3 m, on June 27, 2005 by SO.

Voucher specimen 3, MNHN Zoan.2005.0003, collected from Hakamagoshi, Sakurajima, Kagoshima, Japan (30°35'N, 130°35'E), depth=3 m, on June 27, 2005 by SO; deposited in the Museum National d'Histoire Naturelle, Paris (MNHN).

Voucher specimen 4, USNM 1083157, collected from Hakamagoshi, Sakurajima, Kagoshima, Japan (30°35'N, 130°35'E), depth=3 m, on June 27, 2005 by SO; deposited in the National Museum of Natural History, Smithsonian Institution, Washington D.C. (USNM).

Other material examined. ZSH1 (JAMSTEC # 060380), collected from Hakamagoshi, Sakurajima, Kagoshima, Japan (30°35'N, 130°35'E), depth=5 m, on June 24, 2004 by JDR; ZSH2 (# 060381), collected from Hakamagoshi, Sakurajima, Kagoshima, Japan (30°35'N, 130°35'E), depth=5 m, on June 24, 2004 by JDR; ZSH17 (# 060382), collected from Hakamagoshi, Sakurajima, Kagoshima, Japan (30°35'N, 130°35'E), depth=1 m, on July 17, 2004 by JDR; ZSH23 (# 060383), collected from Hakamagoshi, Sakurajima, Kagoshima, Japan (30°35'N, 130°35'E), depth=9 m, on July 17, 2004 by JDR; ZAT1 (# 060385), collected from Tomori, Kasari, Amami, Kagoshima, Japan (28°27'N, 129°44'E), depth=0 m, on August 8, 2004 by JDR; ZAT2 (# 060386), collected from Tomori, Kasari, Amami, Kagoshima, Japan (28°27'N, 129°44'E), depth=0 m, on August 8, 2004 by JDR; ZAT3 (# 060387), collected from Tomori, Kasari, Amami, Kagoshima, Japan (28°27'N, 129°44'E), depth=0 m, on August 8, 2004 by JDR; ZAT4 (# 060388), collected from Tomori, Kasari, Amami, Kagoshima, Japan (28°27'N, 129°44'E), depth=0 m, on August 8, 2004 by JDR; ZAT5 (# 060389), collected from Tomori, Kasari, Amami, Kagoshima, Japan (28°27'N, 129°44'E), depth=+0.5 m, on August 8, 2004 by JDR; ZAT6 (# 060390), collected from Tomori, Kasari, Amami, Kagoshima, Japan (28°27'N, 129°44'E), depth=1 m, on August 8, 2004 by JDR; ZAT7 (# 060391), collected from Tomori, Kasari, Amami, Kagoshima, Japan (28°27'N, 129°44'E), depth=1 m, on August 8, 2004 by JDR; ZAT11 (# 060392), collected from Tomori, Kasari, Amami, Kagoshima, Japan (28°27'N, 129°44'E), depth=+0.5 m, on August 8, 2004 by JDR (see Table 1 for details); ZYS2 (# 060384), collected from Sangohama, Kurio, Yakushima, Kagoshima, Japan (30°16'N, 130°25'E), depth=2 m, on July 19, 2004 by JDR; 5 samples conserved in JDR's collection from Sangohama, Kurio, Yakushima, Kagoshima, Japan collected by JDR; 5 samples conserved

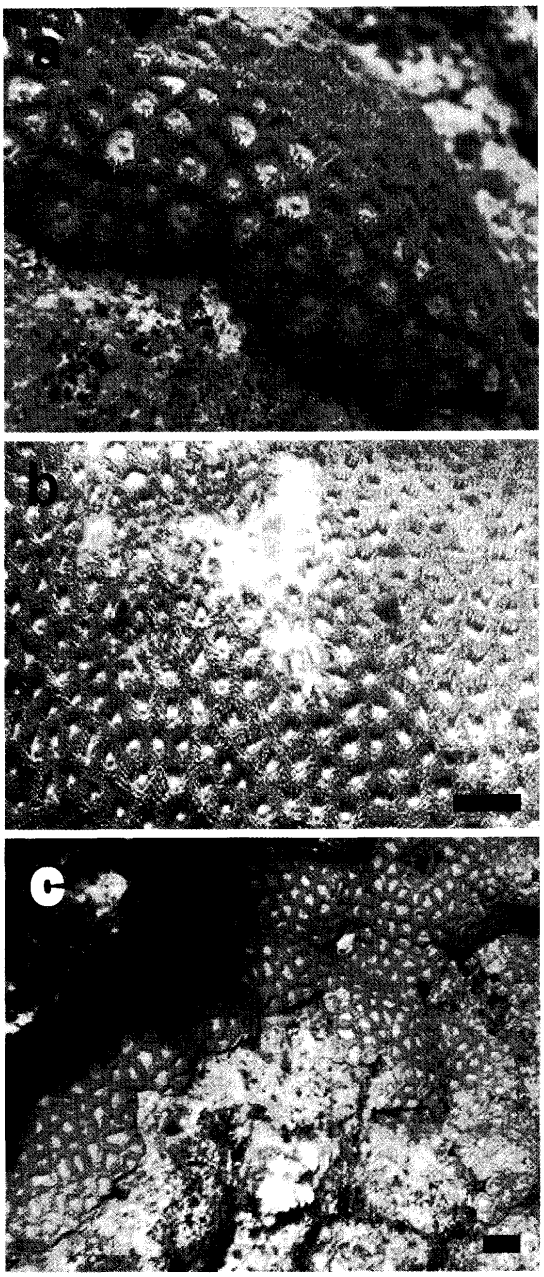


Fig. 3. *Zoanthus kuroshio* n. sp.; samples of different morphotypes used in this study. **a)** sample ZkWK1, collected from Kushimoto, depth=–1.0 m. **b)** holotype, NSMT-CO 1445, Yakushima, –1.5 m. **c)** ZkKK1, Kerama, –8.0 m. White/black bars=1 cm. For sample abbreviations, refer to Table 1.

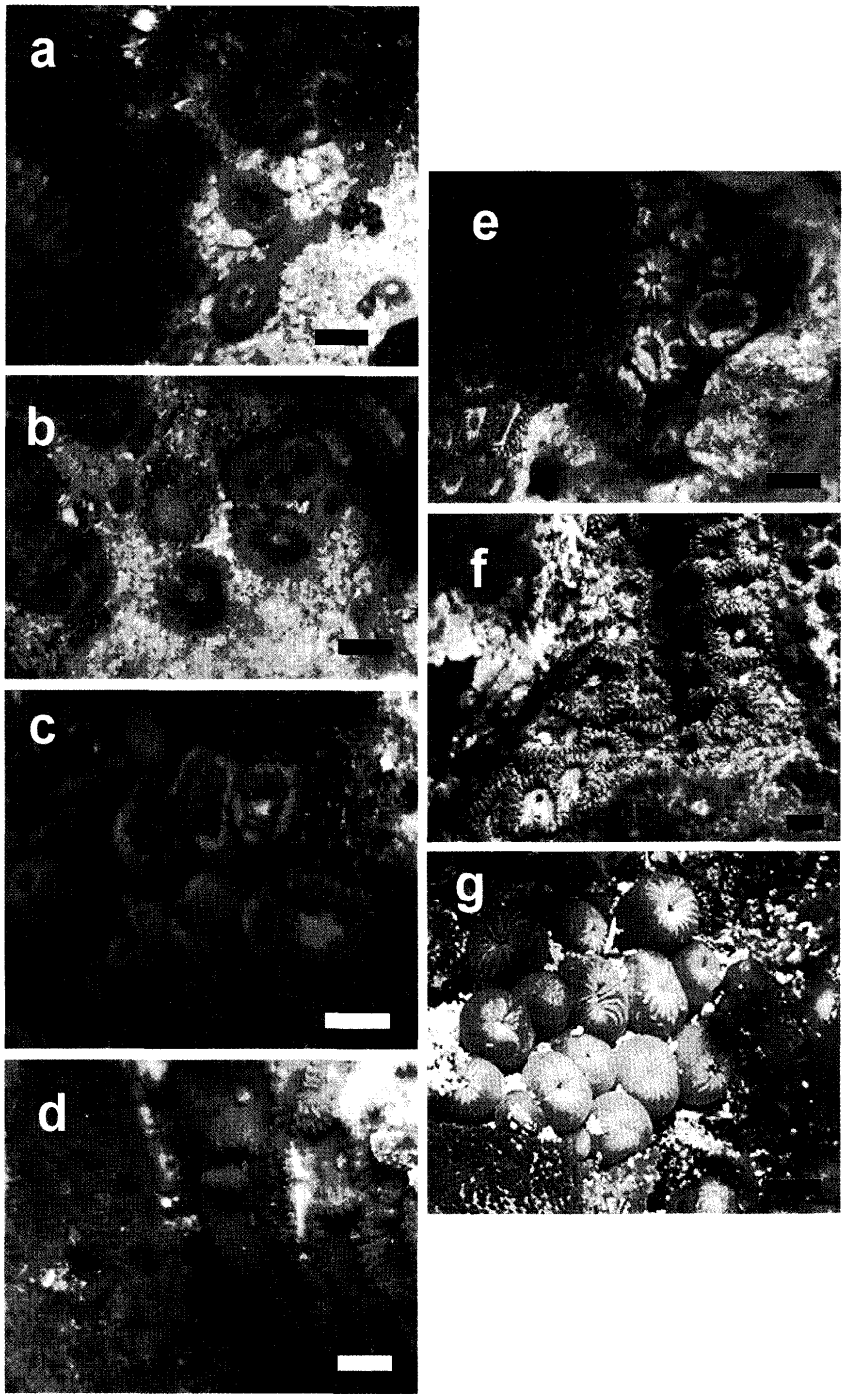


Fig. 4. *Zoanthus gigantus* n. sp.; samples of different morphotypes used in this study. **a)** sample ZgAT1, collected from Amami site, depth=–1.0 m. **b)** ZgAT2, Amami, –1.0 m. **c)** ZgAT8, Amami, –1.0 m. **d)** ZgAT10, Amami, –1.0 m. **e)** ZgYS1, Yakushima, –1.5 m; note white and green *Z. sansibaricus* polyps in lower left corner for size comparison and some closed polyps on right. **f)** holotype NSMT-CO 1443, Yakushima, –3.0 m. **g)** paratype NSMT-CO 1444, Yakushima, –3.0 m, polyps closed. White/black bars=1 cm. For sample abbreviations, refer to Table 1.

in JDR's collection from Tomori, Kasari, Amami, Kagoshima, Japan collected by JDR (see Table 1 samples), and 13 other samples conserved in JDR's collection from Hakamagoshi, Sakurajima, Kagoshima, Japan collected by JDR.

Description. Body wall not encrusting; polyps arise from a lamellate coenenchyme. Adult polyps 3 to 12 mm in diameter (expanded polyps) and 20 mm in length. Mesogleal thickness from 100 to 750 μm, with polyp diameter (closed polyps) 1800–4000 μm in cross-sections and longitudinal sections (Table 2, Fig. 6). Polyps slightly wider in diameter towards the oral opening than at base (Figs. 6e, f; 9). Colony form "liberae" (Pax, 1910), with polyps generally standing free and clear of coenenchyme. External polyp surface light to dark purple with no markings, generally uniform in color (slightly paler around the edge of the oral disk), generally same color as coenenchyme (Fig. 5b, right). Oral disk and tentacles vary widely in color between individual colonies (orange, red, brown, green, purple, white, blue, yellow), sometimes fluorescent (Fig. 2b). Tentacle count 40-58 with, 48-53 mesenteries (Table 2). Can form massive colonies

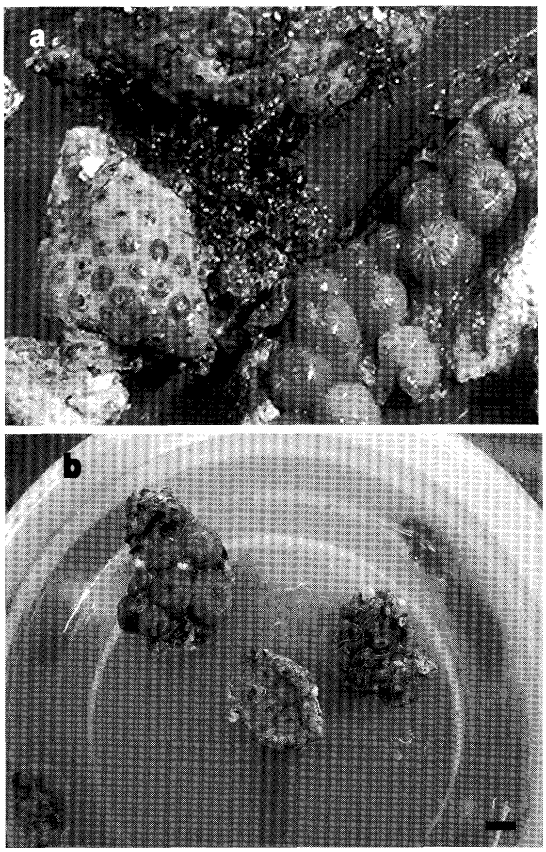


Fig. 5. Comparison of polyp size and form among *Zoanthus* species. **a)** *Z. kuroshio* n. sp. (left), the polyps deeply embedded in a well developed coenenchyme and relatively small, approximately 1800–2500 μm in diameter when closed; *Z. gigantus* n. sp. (right), the polyps loosely connected to a less well developed coenenchyme (basal lamellae, not visible), and larger, 6000–7500 μm diameter. White vertical patterns are visible on the external surface of polyps of *Z. gigantus* n. sp. **b)** Polyps of *Z. gigantus* n. sp. (left), *Z. kuroshio* n. sp. (middle), and *Z. sansibaricus* (right), all from Tomori, Amami. Black bar=1 cm.

(1.5 m^2), intermingled with other color morphotypes of the same species.

Habitat. Found on rocks and coral reef substrates in areas close to strong water currents or large amounts of wave action. Found in shallow subtidal water to depths of 10 m to 15 m, sometimes also intertidal (at Yakushima, Amami, Okinoerabu, Yoron sites).

Distribution. In this study, found at Kushimoto, Kokubu, Sakurajima, Bonotsu, Yakushima, Amami, Okinoerabu, Yoron, all in Japan (Fig. 1). Also found at Lau Lau Beach, Saipan (data not shown).

Remarks. *Zoanthus sansibaricus* as described by Carlgren (1900) and by Uchida (2001) has a blue/purple oral disk with white or yellow mouth. We can be certain that our *Z. sansibaricus* specimens are the species described in Uchida (2001), as we have collected numerous samples of the same morphotype from the same locations as given in Uchida's work. Genetic studies indicate *Z. sansibaricus* is conspecific with *Z. erythrochloros* (Pax and Mueller, 1957), *Z. gnophodes* (ibid), and *Z. pacificus* (Walsh and Bowers, 1971), all described as separate species in Uchida (2001) (see Reimer *et al.*, 2004; unpublished data) (Fig. 2). We have elected to group these four former species into *Z. sansibaricus*, keeping the name *Z. sansibaricus* as it is the oldest binomen. However, it is interesting to note that these four nominal species all possess varying and overlapping morphologies (Fig. 6) – particularly with respect to variable polyp (closed) diameter, mesentery and tentacle count, and highly variable mesogleal thickness. Further genetic analyses may indicate *Z. sansibaricus* includes several other nominal species mentioned in the literature. Additionally, the distribution of *Z. sansibaricus* may be much wider than listed here, as indicated by mt 16S rDNA sequences from samples from Sulawesi, Brazil, and Guam (Fig. 11); however, ITS-rDNA comparisons between these samples are needed, as mitochondrial DNA has been speculated to be highly conserved among Anthozoans (France and Hoover, 2002).

At Sakurajima, *Z. sansibaricus* is a hermaphroditic spawner and has been shown to reproduce sexually annually in synchrony with summer full moons (Ono *et al.*, 2005). In southern Japan, *Z. sansibaricus* has a flexible association with different clades of *Symbiodinium* zooxanthellae, which it acquires horizontally (Reimer *et al.*, 2004, 2006, unpublished data; Ono *et al.*, 2005). It has been shown that *Z. sansibaricus* in southern Japan may possibly hybridize with another, unidentified *Zoanthus* species (Reimer *et al.*, unpublished data).

Genetic sequences. Cytochrome oxidase I: AB128894, AB128897, AB128898 (Reimer *et al.*, 2004); AB194014-AB194036 (supplemental to Reimer *et al.*, 2004); AB214162-AB214174 (Reimer *et al.*, unpublished data). ITS-rDNA: AB214124-AB214149 (Reimer *et al.*, unpublished data). mt 16S rDNA: AF282935, AF282938 (Burnett, unpublished) (possible but not established); AY049069 (Longo *et al.* 2001) (possible but not established) AB219187, AB219188 (this study).

Zoanthus kuroshio **Reimer and Ono, n. sp.**

(Figs. 1, 3, 5, 7, 9; Tables 1, 2)

Material examined. Holotype, NSMT-CO 1445, collected from Sangohama, Kurio, Yakushima, Kagoshima, Japan (30°16'N, 130°25'E), depth=3 m, on April 24, 2005 by JDR. Paratype 1, NSMT-CO 1446, collected from Sangohama, Kurio, Yakushima, Kagoshima, Japan (30°16'N, 130°25'E), depth=3 m, on April 24, 2005 by JDR. Paratype 2, MNHM Zoan.2005.0002, collected from Sangohama, Kurio, Yakushima, Kagoshima, Japan (30°16'N, 130°25'E), depth=3 m, on April 24, 2005 by JDR. Paratype 3, USNM 1083159, collected from Sangohama, Kurio, Yakushima, Kagoshima, Japan (30°16'N, 130°25'E), depth=3 m, on April 24, 2005 by JDR.

Other specimens. ZkWK1 (JAMSTEC # 060393), collected from Kushimoto Marine Park, Kushimoto, Wakayama, Japan (33°28'N, 135°45'E), depth=1 m, on August 3, 2004 by JDR; ZkBA2 (# 060394), collected from Akamizu, Bonotsu, Kagoshima, Japan (31°17'N, 130°13'E), depth=2 m, on August 5, 2004 by JDR; ZkKK1 (# 060396), collected from Kuroshima-kita, Kerama, Okinawa, Japan (26°11'N, 127°30'E), depth=8 m, on June 26, 2004 by JDR (see Table 1 for details).

Diagnosis. Body wall not encrusting; when oral disks are closed polyps crowded and barely protrude from a well-developed, thick, rubbery, lamellate coenenchyme, ("inter-

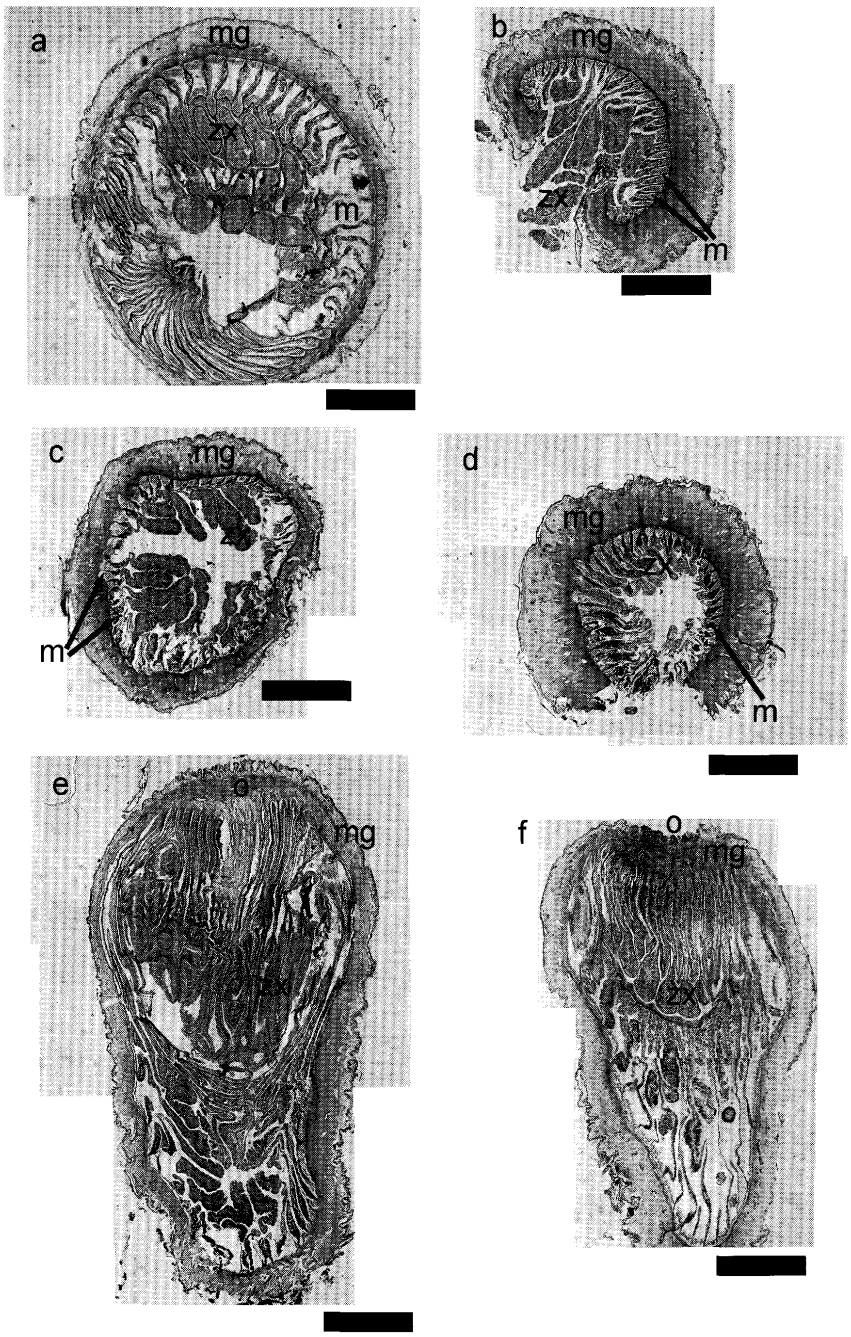


Fig. 6. Sections of *Zoanthus sansibaricus*. **a)** Cross section (XS) of *Z. sansibaricus* aff. *gnophodes*. **b)** XS of *Z. sansibaricus* aff. *pacificus*. **c)** XS of *Z. sansibaricus*. **d)** XS of *Z. sansibaricus*. **e)** longitudinal section (LS) of *Z. sansibaricus* aff. *erythrochloros*. **f)** LS of *Z. sansibaricus* aff. *gnophodes*. All samples collected from the Sakurajima site. Abbreviations: m, mesentery; mg, mesoglea; o, oral opening; zx, zooxanthellae. All black bars=1000 μm.

mediae" or "immersae" (Pax, 1910)) (Fig. 5a, b). Edge of coenenchyme often "tongue-like" in form. Coenenchyme generally lighter in color (pale purple) than polyps. Occasionally external polyp surface around the oral disk edge lighter than the rest of the polyp, almost cream in color (Fig. 5a). External polyp surface light purple with no patterned markings (Fig. 5). Oral disk and tentacles often vary slightly in color (generally pale pink; tentacles pale pink, light green, or gray) between individual colonies (Fig. 3). Expanded polyps 6 to 12 mm in diameter (closed polyps approximately 3 mm in diameter) and up to 7 mm in length. Mesogleal thickness 100 to 1000 μm, with polyp diameter (closed polyps) 700–3500 μm in cross sections and longitudinal sections (Table 2, Fig. 7). Polyps much narrower in diameter towards the oral opening than at the base (Figs. 7b, 9). Tentacle count approximately 50–64, mesentery count approximately 42 to 52. Often in massive colonies (1.5 m^2).

Description of the holotype (Fig. 3b). Partial colony (3 cm×2 cm) consisting of approximately 17 whole polyps, averaging 3 mm in diameter, all connected by a well developed coenenchyme. Oral disk light pink in color, polyps barely extend from coenenchyme (between "immersae" and "intermediae" (Pax, 1910)). Some dead coral substrate is attached to the base of the coenenchyme. Coenenchyme appears as a "lip" on edges, approximately 0.1 cm thick. Holotype has been fixed in Bouin's fluid and then transferred

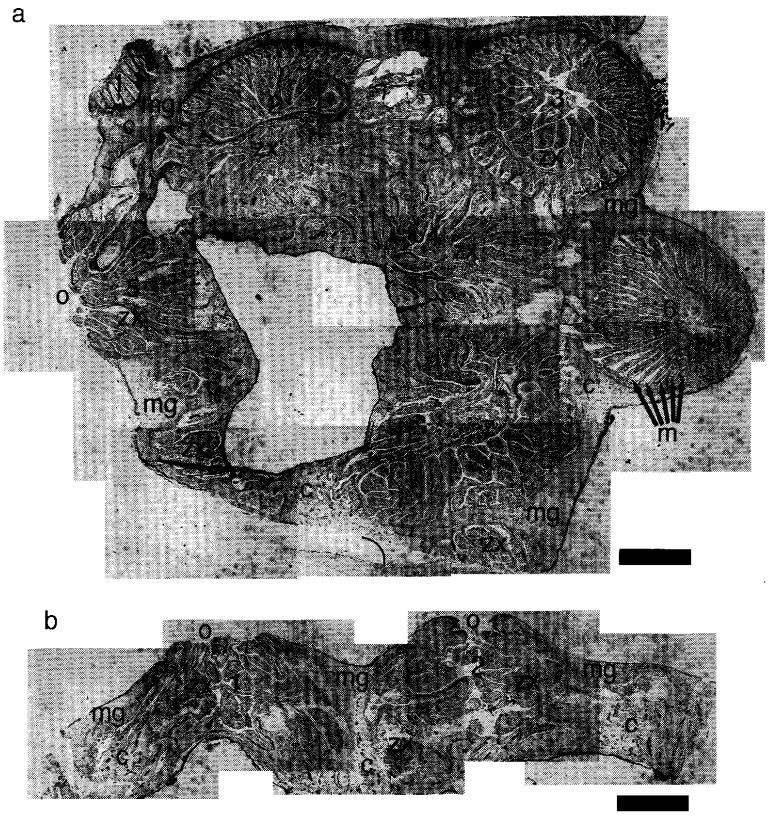
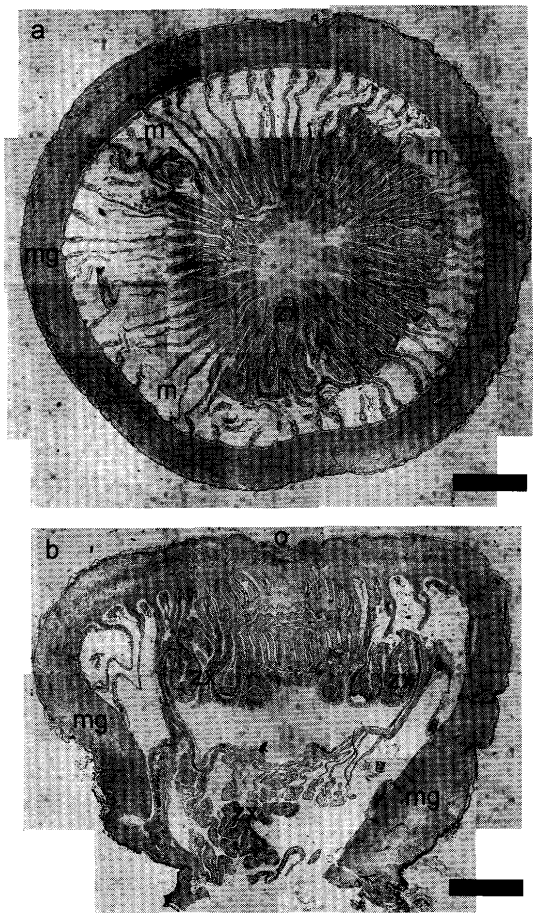


Fig. 7. Sections of *Zoanthus kuroshio* n. sp. **a)** Cross section of *Z. kuroshio* n. sp. showing several polyps (numbers) and a well-developed coenenchyme. **b)** longitudinal section of two polyps (1, 2) and coenenchyme of *Z. kuroshio*. n. sp. Abbreviations: m, mesentery; mg, mesoglea; zx, zooxanthellae; c, coenenchyme; o, oral opening. Note that the coenenchyme consists primarily of mesoglea. All black bars=1000 μm. All sections from holotype colony NSMT-CO 1445.



to 10% formalin.

Etymology. Named for the Black Current (*kuroshio* in Japanese) that flows northeastwardly along the Pacific Coast of Japan where this species is found.

Habitat. Found on dead coral substrate or rocks. Generally found in exposed areas (i.e., reef crests, tops of boulders and rocks) compared to *Z. sansibaricus* and especially *Z. gigantus* n. sp.; can be found from the lower intertidal zone to depths of up to 15 m (Okinawa and Saipan).

Distribution. Has been found at Kushimoto, Yakushima, Amami, Yoron, Okinawa (main island), Kerama Islands (Table 1, Fig. 1), as well as at Uji Islands, Kagoshima, Japan, and Lau Lau Beach and Dimple, Saipan.

Remarks. Paratypes 1 to 3 do not show any significant differences in colony and polyp appearance from the holotype.

It should be noted that the colony and polyp structures of *Z. vietnamensis* (Pax and Mueller, 1957) as described in Burnett *et al.* (1997) strongly resemble those of *Z. kuroshio* n. sp., although oral disk color as described within their study does not. Additionally, genetic sequences obtained from *Z. vietnamensis* (as described in Japan by Uchida, 2001) at Sakurajima are different from those of *Z. kuroshio*

Fig. 8. Sections of *Zoanthus gigantus* n. sp. **a)** Cross section of *Z. gigantus* n. sp. **b)** longitudinal section of *Z. gigantus* n. sp. Abbreviations: m, mesentery; mg, mesoglea; o, oral opening; zx, zooxanthellae. All black bars=1000 μm. All sections from holotype colony NSMT-CO 1443.

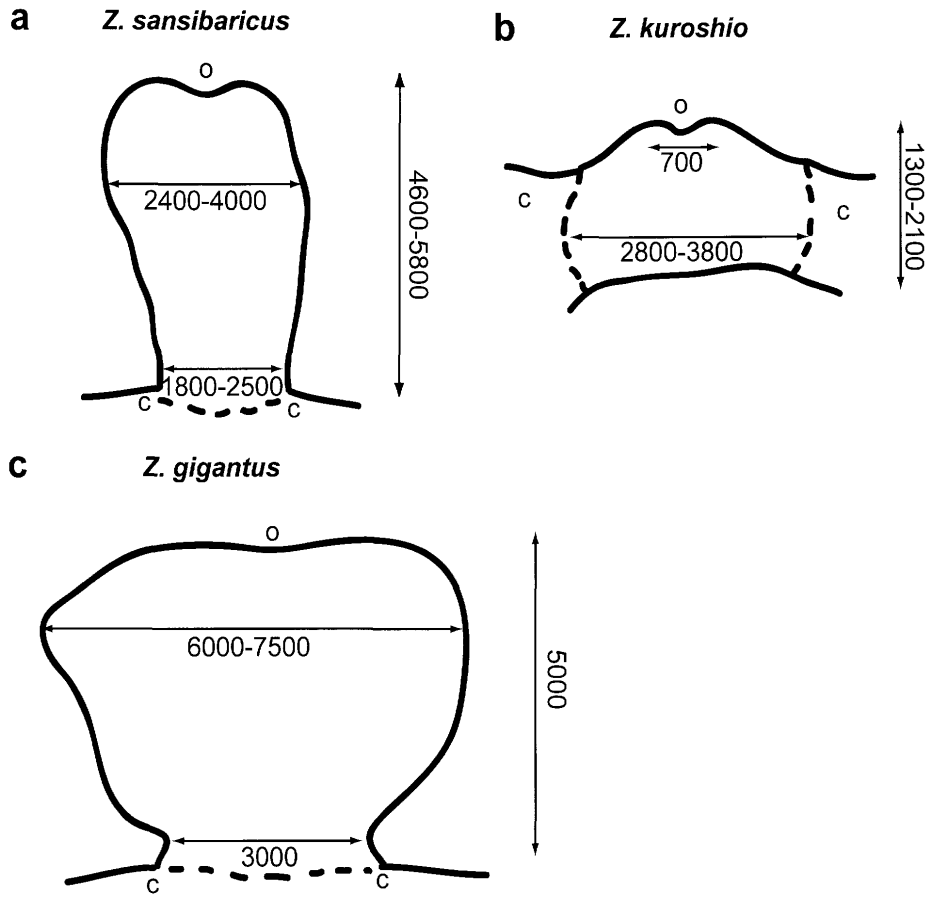


Fig. 9. Generalized sketch of differences in polyp structure and size of the three species of *Zoanthus* in this study. **a)** *Z. sansibaricus*. **b)** *Z. kuroshio* n. sp. **c)** *Z. gigantus* n. sp. Abbreviations: o, oral opening; c, coenenchyme. All sizes in μm.

Table 1. Zoanthid samples used in phylogenetic analyses in the current study.

Sample[c]	Location	Date sampled	Depth (m) below low tide	Oral disk color (inner to outer)	Tentacle #	Oral disk diameter (mm) (open)	COI accession number	16S accession number	Species[d]
SakZsans[a]	Sakurajima	July 03	–3.0	lt. purple, purple	54–56	6–10	AB194031[a]	NA	sansi
SakZery1[a]	Sakurajima	July 03	–3.0	white, red, blue	54	6–10	AB194028[a]	NA	sansi
SakZery2[a]	Sakurajima	July 03	–3.0	white, red, blue	54	6–10	AB194029[a]	NA	sansi
SakZgnop[a]	Sakurajima	July 03	–3.0	white, blue, green	54–56	6–10	AB194030[a]	NA	sansi
SakZpac1[a]	Sakurajima	July 03	–2.0	fl. green	54–58	6–10	AB194016[a]	NA	sansi
SakZpac2[a]	Sakurajima	July 03	–2.0	fl. green	54–58	6–10	AB214162[b]	NA	sansi
SakZ1[a]	Sakurajima	Aug 03	–3.0	green–brown	NA	6–10	AB128894[a]	NA	sansi
SakZ2[a]	Sakurajima	Aug 03	–3.0	green–brown	NA	6–10	AB194021[a]	NA	sansi
SakZ3[a]	Sakurajima	Aug 03	–3.0	yellow	NA	6–10	AB194022[a]	NA	sansi
SakZ4[a]	Sakurajima	Aug 03	–3.0	yellow	NA	6–10	AB194023[a]	NA	sansi
SakZ5[a]	Sakurajima	Aug 03	–3.0	white, red	NA	6–10	AB194024[a]	NA	sansi
SakZ6[a]	Sakurajima	Aug 03	–3.0	yellow	NA	6–10	AB194025[a]	NA	sansi
SakZ7[a]	Sakurajima	Aug 03	–3.0	white	NA	6–10	AB194026[a]	NA	sansi
ZSH1[b]	Sakurajima	June 04	–5.0	lt. fl. green, dk. fl. green	48	6–8	AB214163[b]	NA	sansi
ZSH2[b]	Sakurajima	June 04	–5.0	lt. fl. green, dk. fl. green	48	6–8	AB214164[b]	NA	sansi
ZSH17[b]	Sakurajima	July 04	–1.0	lt. fl. green	NA	6–8	AB214165[b]	NA	sansi
ZSH23[b]	Sakurajima	July 04	–9.0	white, dk. purple, fl. green	56	6–8	AB214166[b]	AB219187	sansi
NSMT-CO 1441 (neotype)	Sakurajima	July 05	–3.0	lt. purple, purple	48–54	6–10	NA	NA	sansi
NSMT-CO 1442 (neoparatype)	Sakurajima	July 05	–3.0	lt. purple, purple	48–54	6–10	NA	NA	sansi
MNHN Zoan.2005.0003 (neoparatype)	Sakurajima	July 05	–3.0	lt. purple, purple	48–54	6–10	NA	NA	sansi

Table 1. Continued.

USNM 1083157 (neoparatype)	Sakurajima	July 05	–3.0	lt. purple, purple	48–56	6–10	NA	NA	sansi
YakuZpac1[a]	Yakushima	Aug 03	+1.0	fl. green	54–58	6–10	AB194017[a]	NA	sansi
YakuZpac2[a]	Yakushima	Aug 03	+1.0	fl. green	54–58	6–10	AB194018[a]	NA	sansi
YakuZ1[a]	Yakushima	Aug 03	+1.5	fl. green, green	NA	6–8	AB194032[a]	NA	sansi
YakuZ2[a]	Yakushima	Aug 03	–1.0	fl. green, white, green	NA	8–12	AB194033[a]	NA	sansi
YakuZ5[a]	Yakushima	Aug 03	–1.0	white, purple	NA	8–12	AB194034[a]	NA	sansi
ZYS2	Yakushima	July 04	–2.0	white, light blue, red tentacles	52	6–8	NA	NA	sansi
Amami Zpac1[a]	Amami	Aug 03	+0.5	fl. green	54–58	6–10	AB194020[a]	NA	sansi
Amami Zpac2[a]	Amami	Aug 03	+0.5	fl. green	54–58	6–10	AB194019[a]	NA	sansi
AmamiZ1[a]	Amami	Aug 03	0.0	white, red, blue	NA	4–6	AB128897[a]	NA	sansi
AmamiZ2[a]	Amami	Aug 03	+0.5	white, purple	NA	4–8	AB194035[a]	NA	sansi
AmamiZ5[a]	Amami	Aug 03	+2.0	pink	NA	4–8	AB194036[a]	NA	sansi
ZAT1[b]	Amami	Aug 04	0.0	lt. green, fl. green	54–56	6–8	AB214167[b]	NA	sansi
ZAT2[b]	Amami	Aug 04	0.0	white, purple	48–50	6–8	AB214168[b]	NA	sansi
ZAT3[b]	Amami	Aug 04	0.0	lt. purple, purple	48–52	3–6	AB214169[b]	NA	sansi
ZAT4[b]	Amami	Aug 04	0.0	lt. yellow, lt. gray–green, gray green	40–48	3–6	AB214170[b]	NA	sansi
ZAT5[b]	Amami	Aug 04	+0.5	white, lt. pink	50	3–6	AB214171[b]	NA	sansi
ZAT6[b]	Amami	Aug 04	–1.0	fl. green	54	6–8	AB214172[b]	NA	sansi
ZAT7[b]	Amami	Aug 04	–1.0	purple, orange, purple	44	6–8	AB214173[b]	AB219188	sansi
ZAT11[b]	Amami	Aug 04	+0.5	white, purple	NA	3–6	AB214174[b]	NA	sansi
ZkWK1	Kushimoto	Aug 04	–1.0	lt. green, lt. pink w/ white stripes	50–56	6–12	AB219182	AB219189	kuro
ZkBA2	Bonotsu	Aug 04	–2.0	white, pink	NA	6–8	NA	AB219190	kuro
NSMT-CO 1445 (holotype) [b]	Yakushima	July 04	–1.5	white, pink	58	6–8	AB214175[b]	AB219191	kuro
NSMT-CO 1446 (paratype)	Yakushima	Apr 05	–1.5	white, pink	52	6–8	NA	NA	kuro
MNHN Zoan.2005.0002 (paratype)	Yakushima	Apr 05	–1.5	white, pink	52	6–8	NA	NA	kuro
USNM 1083159 (paratype)	Yakushima	Apr 05	–1.5	white, pink	52	6–8	NA	NA	kuro
ZkAT9[b]	Amami	Aug 04	–1.0	white, purple, pink	50–52	6–8	AB214176[b]	NA	kuro
ZkKK1	Kerama	June 04	–8.0	white, lt. pink	54–64	6–8	AB219183	NA	kuro
ZgYS1[b]	Yakushima	July 04	–1.5	lt. fl. green, dk. fl. green	42–56	12–20	AB214177[b]	AB219192	giga
NSMT-CO 1443 (holotype)	Yakushima	Apr 05	–3.0	fl. green, dk. green, lt. green	44–60	12–25	NA	NA	giga
NSMT-CO 1444 (paratype)	Yakushima	Apr 05	–3.0	fl. green, dk. green, lt. green	44–60	12–25	NA	NA	giga
MNHN Zoan.2005.0001 (paratype)	Yakushima	Apr 05	–3.0	fl. green, dk. green, lt. green	44–60	12–25	NA	NA	giga
USNM 1083158 (paratype)	Yakushima	Apr 05	–3.0	fl. green, dk. green,	44–60	12–25	NA	NA	giga
AmamiZ4[a]	Amami	Aug 03	+1.0	fl. green	NA	10–20	AB128893[a]	NA	giga
ZgAT1	Amami	Aug 04	–1.0	fl. green, dk. green	54–58	10–20	NA	NA	giga
ZgAT2	Amami	Aug 04	–1.0	fl. green, purple	48–60	10–20	AB219184	AB219193	giga
ZgAT8	Amami	Aug 04	–1.0	fl. green, dk. green	48–58	8–16	AB219185	NA	giga
ZgAT10	Amami	Aug 04	–1.0	fl. green, purple, red-brown	54–56	6–16	AB219186	NA	giga
YakuPaly[a]	Yakushima	Aug 03	–1.0	NA	30–32	10–20	AB128896[a]	NA	paly
AmamiPaly[a]	Amami	Aug 03	+2.0	white, green, brown	29–30	10–20	AB128895[a]	NA	paly
Parazoanthus gracilis[b]	Izu	Nov 04	–17.0	yellow	32	3–6	AB214178[b]	AB219194	para

NA=data not obtained or available.

[a] sample from or derived from Reimer *et al.* (2004), and follows nomenclature used within that paper.

[b] sample and/or sequence from Reimer *et al.* (unpublished data). Sequences with no superscript after GenBank Accession Numbers have been obtained in the current study.

[c] Samples are deposited in the National Science Museum of Tokyo (NSMT), the Museum National d'Histoire Naturelle (Paris) (MNHN), the Smithsonian National Museum of Natural History (Washington, D.C.) (USNM), JAMSTEC, or in the collection of JDR (other sample names).

[d] Species abbreviations: sansi=*Z. sansibaricus*, kuro=*Z. kuroshio*, giga=*Z. gigantus*, paly=*Palythoa* spp., and para=*Parazoanthus* sp.

Table 2. Summary of morphological characteristics of sections of *Zoanthus sansibaricus*, *Z. kuroshio*, and *Z. gigantus*.

Species	Section[a]	Mesogleal thickness (μm)[b]	Polyp diameter (μm)	Polyp height (μm)	Mesentery number
Z. sansibaricus aff. *gnophodes*	XS	100–400 (150)	3700–4000	NA	51–52 52–53[c]
aff. *pacificus*	XS	350–600 (500)	2400–2700	NA	52[c]
aff. *sansibaricus*	XS	150–750 (400)	2700–2900	NA	48–50 52[c]
aff. *gnophodes*	LS	200–600 (250)	1800 (base) 2900 (top)	4600	52–53[c]
aff. *erythrochloros*	LS	100–500 (200)	2500 (base) 3250 (top)	5800	53[c]
Z. kuroshio	XS	100–1000 (250)	2800–3800	NA	42–43
Z. kuroshio	LS	100–300 (150)	3500 (base) 700 (top)	1300–2100	NA
Z. gigantus	XS	400–700 (500)	6000–7500	NA	62–63
Z. gigantus	LS	300–1000 (500)	3000 (base) 6500 (top)	5000	NA

[a] abbreviations: XS=cross section, LS=longitudinal section.
[b] minimum and maximum, with average in parentheses.
[c] from Reimer *et al.* (2004).

n.sp. (Reimer, unpublished data)

Based on mt 16S rDNA sequence results, *Z. kuroshio* may also be present in Cape Verde and Panama (Fig. 11), although as with *Z. sansibaricus* other sequence data from more genetic markers is needed to confirm or deny this.

Genetic sequences. Cytochrome oxidase I, AB214175, AB214176 (Reimer *et al.*, unpublished data); AB219182, AB219183 (this study). ITS-rDNA, AB214159, AB214160 (Reimer *et al.*, unpublished data). mt 16S rDNA, AF282933, AF282934 (Burnett unpublished) (possible but not established); AB219189~AB219191 (this study)

Zoanthus gigantus **Reimer and Tsukahara, n. sp.**

(Figs. 1, 4, 5, 8, 9; Tables 1, 2)

Material examined. Holotype, NSMT-CO 1443, collected from Sangohama, Kurio, Yakushima, Kagoshima, Japan (30°16'N, 130°25'E), depth=3 m, on April 24, 2005 by JDR. Paratype 1, NSMT-CO 1444, collected from Sangohama, Kurio, Yakushima, Kagoshima, Japan (30°16'N, 130°25'E), depth=3 m, on April 24, 2005 by JDR. Paratype 2, MNHN Zoan.2005.0001, collected from Sangohama, Kurio, Yakushima, Kagoshima, Japan (30°16'N, 130°25'E), depth=3 m, on April 24, 2005 by JDR. Paratype 3, USNM 1083158, collected from Sangohama, Kurio, Yakushima, Kagoshima, Japan (30°16'N, 130°25'E), depth=3 m, on April 24, 2005 by JDR.

Other specimens. ZgYS1 (JAMSTEC # 060397), collected from Sangohama, Kurio, Yakushima, Kagoshima, Japan (30°16'N, 130°25'E), depth=1.5 m, on July 18, 2004 by JDR; ZgAT1 (# 060398), collected from Tomori, Kasari, Amami, Kagoshima, Japan (28°27'N, 129°44'E), depth=1 m, on August 8, 2004 by JDR; ZgAT2 (# 060399), collected from Tomori, Kasari, Amami, Kagoshima, Japan (28°27'N, 129°44'E), depth=1 m, on August 8, 2004 by JDR; ZgAT8 (# 060400), collected from Tomori, Kasari, Amami, Kagoshima, Japan (28°27'N, 129°44'E), depth=1 m, on August 8, 2004 by JDR; ZgAT10 (# 060401), collected from Tomori, Kasari, Amami, Kagoshima, Japan (28°27'N, 129°44'E), depth=1 m, on August 8, 2004 by JDR (see Table 1 for details). Also one other sample conserved in JDR's collection from Tomori, Kasari, Amami, Kagoshima, Japan (see Table 1 sample with no JAMSTEC or museum sample number).

Diagnosis. Body wall not encrusting; polyps extend from a poorly developed lamellate coenenchyme and not crowded, i.e., "liberae" form (Pax, 1910) (Fig. 4b). Expanded polyps from 6 to 25+ mm in diameter, up to 30–40 mm in length (Fig. 5). External polyp surface purple with cream/white striped vertical markings on the upper half (Figs. 4e, g). Oral disk and tentacles vary in color (green, brown, gray, blue, red), sometimes fluorescent (Fig. 4a). Tentacle count approximately 42 to 60, with 62-63 mesenteries. Mesogleal thickness from 300 to 1000 μm, with polyp diameter (closed polyps) 3000–7500 μm in cross sections and longitudinal sections (Table 2, Fig. 8). Polyps over twice as wide in diameter towards the oral opening as at the base (Figs. 8b, 9). Forms small colonies of 2-50 polyps, occasionally solitary (seen at the Amami and Kerama sampling sites).

Description of the holotype (Fig. 4f). Partial colony (10 cm in length×1–3 cm in width) consisting of approximately 16 to 17 whole polyps, 0.5 to 1.0 cm in diameter and 0.5 to 1.5 cm in length, all loosely connected by a coenenchyme. Oral disk light green with darker green central disk, and very light green/cream oral opening. Some dead coral substrate is attached to the base of the coenenchyme. External polyp surface is purple with 10–20 thin white stripes (<500 μm width) running vertically from the oral opening to approximately the area of the widest diameter of the polyp below

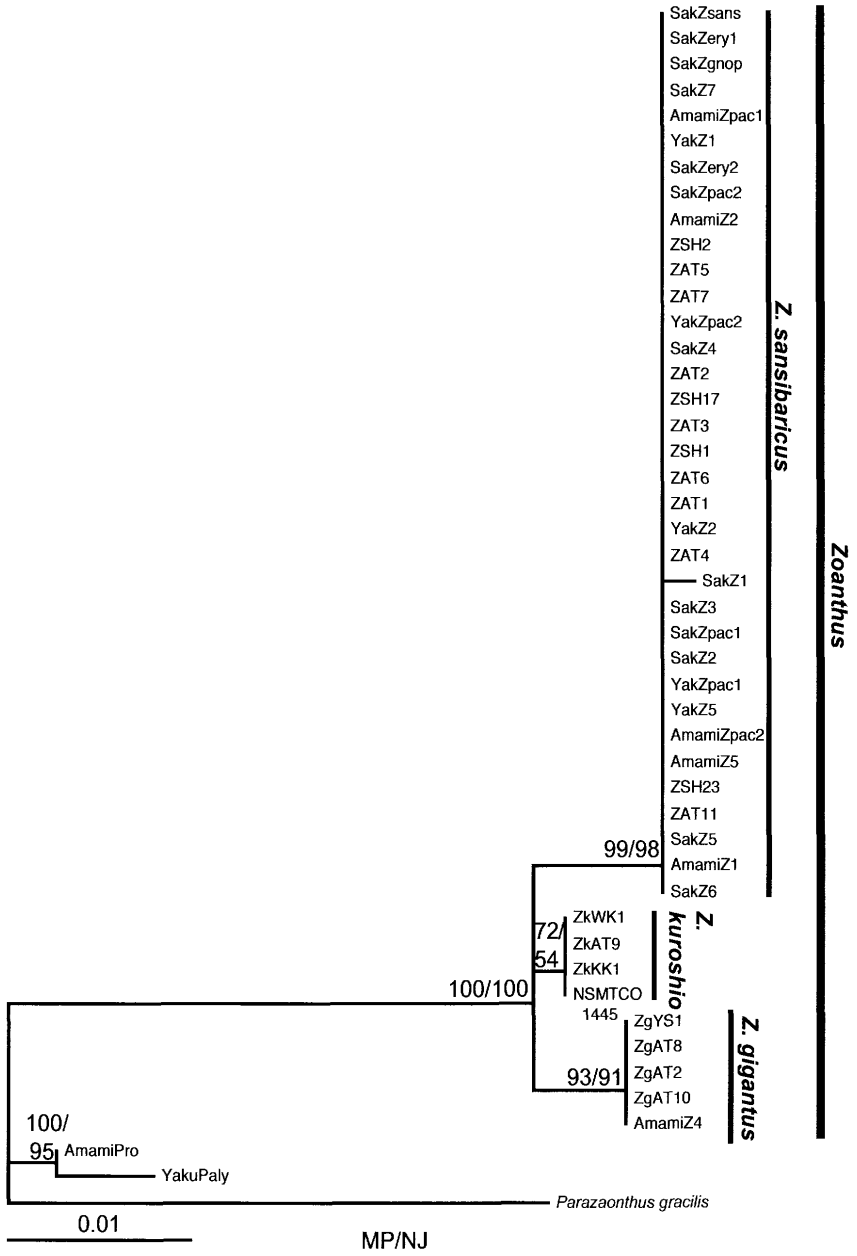


Fig. 10. Maximum likelihood tree from analysis of mitochondrial cytochrome oxidase I gene (COI) sequences. Values at branches represent ML and NJ bootstrap probability, respectively (>50%). For sample name abbreviations, refer to Table 1.

the oral opening, with the total length of the stripes approximately 1500–2000 μm (similar to Fig. 4g). Holotype has been fixed in Bouin's fluid and stored in 10% formalin.

Etymology. Named for its size when compared to other *Zoanthus* species.

Habitat. Found in shallow subtidal waters (<5 m) on dead coral substrate, usually with coenenchyme in shaded cracks or buried beneath loose sand.

Distribution. Yakushima, Amami (Fig. 1), as well as Yoron (observation only, JDR), Kagoshima, Japan, Shirahama, Wakayama, Japan (observation only, JDR), and Kerema, Okinawa, Japan.

Remarks. Molecular studies indicate *Z. gigantus* n. sp. may have an as of yet undetected sibling species that hybridizes with *Z. sansibaricus* at the Yakushima and Amami sampling locations (Reimer *et al.*, unpublished data). Paratypes show polyps of variable length (from 0.3 to 1.0 cm) but no other notable morphological differences from the holotype, including oral disk color.

Genetic sequences. Cytochrome oxidase I, AB128893 (Reimer *et al.*, 2004), AB214177 (Reimer *et al.*, unpublished data), AB219184-AB219186 (this study). ITS-rDNA, AB214123, AB214158 (Reimer *et al.*, unpublished data). mt 16S rDNA, AB219192, AB219193 (this study).

KEY TO SPECIES

1. White vertical stripes on external surface of closed polyp; polyp diameter towards oral disk usually over twice as wide as polyp diameter at base – *Zoanthus gigantus* n. sp.
2. No striped markings on external surface of closed polyp – go to 3 and 4.
3. Coenenchyme well-developed, thick and rubbery; polyps barely extend past coenenchyme, much narrower in

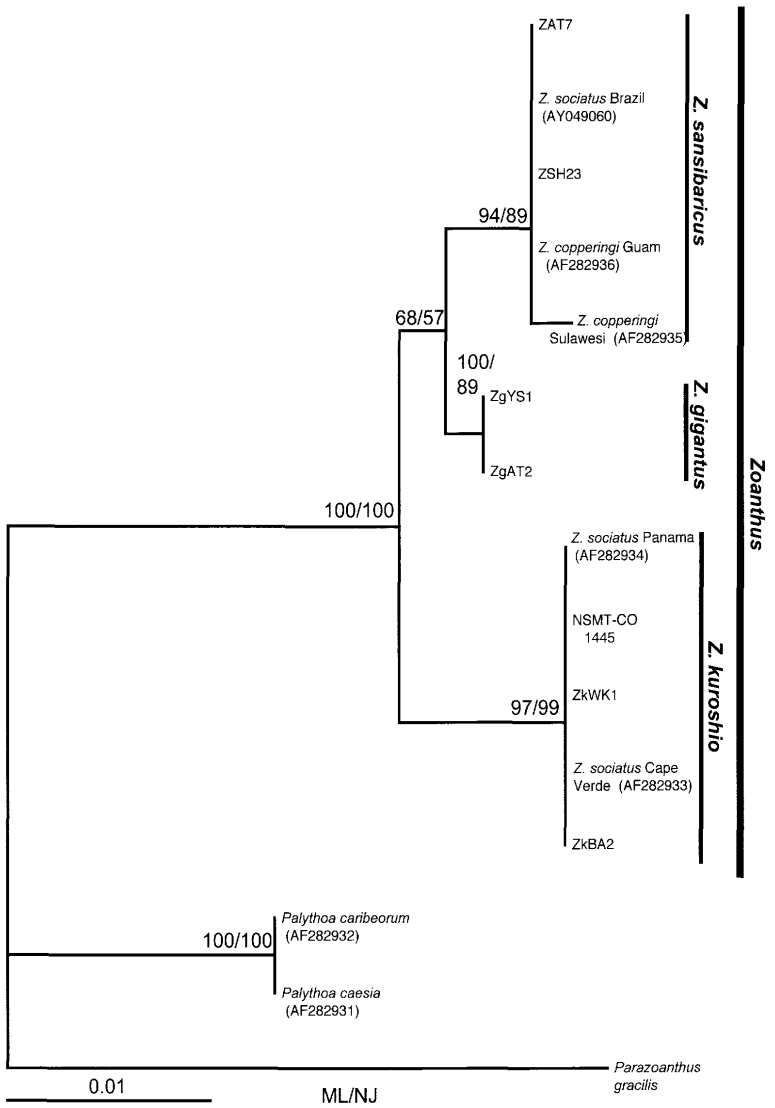


Fig. 11. Maximum likelihood tree from analysis of mitochondrial 16S rDNA sequences. Values at branches represent ML and NJ bootstrap probability, respectively (>50%). For sample name abbreviations, refer to Table 1.

diameter at oral opening than at base in sections – *Zoanthus kuroshio* n. sp.

4. Polyps extend out from coenenchyme, almost the same in diameter throughout the length of the polyp, or slightly larger towards the oral disk – *Zoanthus sansibaricus.*

MOLECULAR RESULTS

Sequences and phylogeny of the COI gene

The ML tree based on the COI sequences is shown in Fig. 10. All assumed *Z. sansibaricus* samples formed a monophyletic group with strong ML bootstrap support (99%). Similarly, all nominal *Z. gigantus* n. sp. formed a distinct monophyletic group, again with high bootstrap support (93%). ML bootstrap support was not so high for *Z. kuroshio* n. sp. (72%), but as our *Z. kuroshio* n. sp. alignment showed clear species-specific substitutions we are confident in the phylogeny presented here. Overall, *Z. kuroshio* n. sp. COI sequences differed from *Z. sansibaricus* by 0.7% (4/595 bp), and *Z. gigantus* n. sp. COI sequences differed from *Z. sansibaricus* by 1.0% (6/595 bp).

Sequences and phylogeny of mt 16S rDNA

The ML tree based on the mt 16S rDNA sequences is shown in Fig. 11. Overall the topology was very similar to the COI topology, with the only notable difference being that *Z. gigantus* n. sp. and *Z. sansibaricus* formed a clade separate from *Z. kuroshio* n. sp. All assumed *Z. sansibaricus* samples formed a monophyletic group with strong ML bootstrap support (94%). Samples of Brazilian *Z.* aff. *sociatus* (AY049060), and *Zoanthus* aff. *coppingeri* from Guam (AF282936) and Sulawesi (AF282935), were included in the *Z. sansibaricus* clade. Similarly, all nominal *Z. kuroshio* n. sp. and *Z. gigantus* n. sp. formed separate monophyletic groups, again with high ML bootstrap support (97% and 100%, respectively). Putative *Z.* aff. *sociatus* sequences from Cape Verde (AF282933) and Panama (AF282934) were within the *Z. kuroshio* n. sp. clade. Overall, *Z. kuroshio* n. sp. COI sequences differed from *Z. sansibaricus* by 1.7% (9/532 bp), and *Z. gigantus* n. sp. COI sequences differed from *Z. sansibaricus* by 0.9% (5/532 bp).

DISCUSSION

What is the true level of species diversity in *Zoanthus*?

All *Zoanthus* mt 16S rDNA sequences from previous investigations clustered with either our *Z. sansibaricus* samples or in the *Z. kuroshio* n.sp. clade. Additionally, initial COI sequence results from *Zoanthus* samples from Bali, Indonesia, and Saipan again show all samples examined belonging to either the *Z. sansibaricus* or the *Z. kuroshio* n.sp. clade (JDR, data not shown). Although this possibly indicates that these two species of *Zoanthus* may be worldwide in distribution, and that many species mentioned in literature from various locations may in fact be conspecific, care should be taken interpreting these results in such a fashion. Mitochondrial DNA in Anthozoa has been shown to be very conservative (France and Hoover, 2002), and confirmation of *Zoanthus* species should utilize both mitochondrial and faster evolving markers (such as ITS-rDNA; Reimer *et al.*, unpublished data). The monophyly of all three *Zoanthus* spp. discussed here have been further confirmed by ITS-rDNA sequencing (Reimer *et al.*, unpublished data).

It should be noted that many undescribed species with more limited ranges (such as *Z. gigantus* n.sp. here) may not have yet been described. Our results highlight the confusion surrounding the true level of *Zoanthus* species diversity (with putative *Z.* aff. *sociatus* samples in both the *Z. sansibaricus* and the *Z. kuroshio* n.sp. clades), and the danger of making hasty identifications based solely on morphological data. It remains to be seen whether *Zoanthus* spp. diversity is lower than currently estimated, as this study and recent investigations seem to indicate (Burnett *et al.*, 1995, 1997; Reimer *et al.*, 2004).

A potential taxonomic system for identifying *Zoanthus* species?

Currently there are no standardized taxonomic systems in place for identifying and discerning between various zoanthid species, particularly in the widespread genus *Zoanthus*. While various morphological methods have been used in trying to distinguish species, none have proven to be both a) consistently accurate and b) relatively simple to perform, especially in the field.

Unfortunately, there appears to be no "easy" method for *Zoanthus* identification, especially without comparison between different species. As shown here by the wide morphological variation in *Z. sansibaricus* (with highly variable oral disk color, mesogleal thickness, and polyp diameter), a single *Zoanthus* species can evidently encompass a wide range of morphotypes. This observation reflects data presented by Burnett *et al.* (1997), who suggested zoanthid species have considerable intraspecific morphological and environmental tolerance variation. Tentacle count methods are inaccurate in that the numbers of tentacles and mesenteries increases as the age of the polyp increases. Indeed, we have seen polyps of *Z. sansibaricus* with as few as 40 and as many as 58 tentacles (Table 1). Mesentery count data show less variation and may be an accurate species character, but acquiring accurate data requires the making of numerous delicate cross-section slides. Distinguishing between a "fully-grown" polyp and a "juvenile" polyp is also difficult, as polyp size appears to be morphologically plastic, influenced by not only age but also the location of the colony (*i.e.*, shaded or not, water current, *etc.*) (Karlson, 1988). Thus, a mature polyp in one colony may smaller than a juvenile in another. Similarly, nematocyst morphology, which may be accurate as utilized in *Protopalythoa* spp. (Ryland and Lancaster, 2003, 2004), depends on colony size, the age of the polyp, and the sampling location within the polyp, and thus leaves much room for experimental error. Burnett *et al.* (1997) similarly reported that using nematocysts for identification of *Zoanthus* species was unsuccessful. Oral disk color, used by some researchers to distinguish between *Zoanthus* species *(i.e.* Uchida, 2001), has been shown to be polyphenetic in other Hexacorallia (Kelmanson and Matz, 2003), and, based on Reimer *et al.* (2004) and our data here (Table 1), oral disk color appears to be non-species-specific in the case of *Z. sansibaricus* and *Z. gigantus* n.sp. However, oral disk color may be a useful indicator of *Z. kuroshio* n.sp., as all the *Z. kuroshio* n.sp. samples we have observed thus far have a light pink oral disk (Table 1) and appear to largely lack the fluorescent coloration seen in *Z. sansibaricus* and *Z. gigantus* n.sp. Polyp external surface color, while appearing to be an accurate character (*Z. kuroshio* n.sp. having lighter coloration than both *Z. gigantus* n.sp. and *Z. sansibaricus*), has been shown to seasonally vary in *Z. sansibaricus* at Sakurajima (SO, data not shown), and identification based solely on this characteristic should be treated with caution.

Polyp external surface pigmentation patterns, as well as, colony and polyp structure appear to offer the most promise as easy-to-use, species-level morphological characters, despite some morphological plasticity in these characteristics. All three species seen in this study can be distinguished from one another by comparative analyses of coenenchyme and polyp structure and the presence or lack of polyp surface pigmentation patterns. Whether these characteristics can be used to distinguish between other *Zoanthus* species and specimens remains to be investigated.

Although we have here presented a species identification key describing morphological characteristics of three *Zoanthus* species, it should be noted that all of our samples are from environments with strong currents (*i.e.*, Sakurajima) and/or wave action *(i.e.*, Amami and Yakushima). We did not find any *Zoanthus* spp. in areas with low water-flow characteristics, although it appears that *Zoanthus* exists in such areas in Indonesia and perhaps other regions (JDR, data not shown). It remains to be seen if the three species observed here live in low water flow and other environments, and if so, how their morphology is affected (*e.g.*, longer polyps, *etc.*). Thus, our morphological species identification key, while accurate for the strong current/wave environments in this study, has not been tested in other environments and should be used with caution.

Molecular analysis of DNA is the most certain method of identifying *Zoanthus* species. The species-level markers COI and mt 16S rDNA used here and in other studies (Reimer *et al.*, 2004, unpublished data; Sinniger *et al.*, 2005) appear to be good markers for *Zoanthus*. Some nuclear markers, such as the internal transcribed spacer region of ribosomal DNA (ITS-rDNA), while possessing a much faster evolutionary rate that COI and mt 16S rDNA (Medina *et al.*, 1999), appear to be too highly divergent from species to

species to be used accurately for phylogenetic studies. *Zoanthus* congeners have been shown to have virtually unalignable ITS-rDNA sequences (Reimer *et al.*, unpublished data). ITS-rDNA length may be an accurate method to identify genotypes, but due to possible species-species hybridization in *Zoanthus*, ITS-rDNA alone should not be utilized to identify *Zoanthus* species (Reimer *et al.*, unpublished data).

As shown in this study, the initial use of genetic data from valid species-level markers to ascertain the identity of putative species, followed by identification of species-specific morphological characters, appears to be the most accurate method of *Zoanthus* species identification.

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